

Consumer and
Corporate Affairs CanadaContinental
of Canada

(11) (A) No. 1 271 708

(45) ISSUED 900717

(52) CLASS 167-130

(51) INT. CL. A61K 39/205

(19) (CA) **CANADIAN PATENT** (12)(54) Process for the Preparation of a Rabies Vaccine and
the Vaccine Obtained by This Process(72) Glück, Reinhard;
Germanier, René;
Switzerland(73) Granted to Schweizerisches Serum- und Impfinstitut
und Institut zur Erforschung der
Infektionskrankheiten, Switzerland

(21) APPLICATION No. 917,612

(22) FILED 860909

(30) PRIORITY DATE (CH) Switzerland (04 999/B3-9)
851122

No. OF CLAIMS 38 - NO DRAWING

Canada

ESTABLISHED BY THE PATENT OFFICE, OTTAWA
CANADA

ABSTRACT OF THE DISCLOSURE

The invention relates to a process for the preparation of a rabies vaccine by multiplication of rabies viruses in animal nerve tissue or poultry embryos, harvesting of the
5 viruses from the nerve tissue or from the heads of the embryos, enriching the viral preparation, inactivating thereof, and preparing a vaccine. In one aspect, the process comprises homogenizing the nerve tissue or embryo heads and harvesting of the viruses therefrom by avoiding the use of a
10 mixer, and thus preventing damage to and fragmentation of the viruses. The nerve tissue, the embryo heads or their contents are comminuted in a manner which preserves cell and viral integrity. The preparation is then treated by separating the complete live viruses which are capable of
15 multiplication from the resulting cell suspension, delipidating by extraction with a water-immiscible organic solvent and then further selectively concentrating the viruses thereof.

The invention also relates to a myelin-free rabies
20 vaccine which has been obtained by the process described above from animal nerve tissue or poultry embryo head tissue containing rabies viruses.

1271708

BACKGROUND OF THE INVENTIONField of the Invention

The present invention relates to a new, economic process for obtaining a rabies vaccine comprising obtaining whole
5 live viruses and rendering the viruses thereof incapable of replicating by chemical treatment. This invention also relates to a vaccine obtained by this process, which is by reason of its high purity, distinguished by a high specific activity and the absence of undesirable secondary reactions
10 when inoculated to human subjects.

Description of the Background

Most rabies vaccines have up until the present time been obtained by multiplication of the rabies virus in living animals such as mice, rats, rabbits, sheep, etc. However,
15 the thus obtained virus-containing preparations contain considerable amounts of myelin and elicit detrimental side effects.

In recent times, rabies vaccines have also been obtained from viruses multiplied in poultry embryos. This method has
20 the advantage, in principle, that the thus obtained virus-containing tissue contains hardly any injurious myelin. After multiplication of the viruses in poultry embryos, these embryos are completely homogenized in toto in a mixer or blender. In this manner, however, it is only possible to
25 incompletely separate from this pasty homogenate the virus

- 2 -

1271708

constituents from heterologous protein which may initiate
undesired secondary reactions upon inoculation. This is also
the case with vaccines obtained from brains of living
animals which have been infected with rabies. On repeated
5 inoculation - indispensable in the case of hunters, forestry
workers, veterinarians, etc. - these secondary reactions may
increase considerably and result in violent allergic defense
reactions against the heterologous proteins.

The quality of embryo vaccines has been somewhat
10 improved by using only the heads of the embryos to obtain the
vaccines. Since, in comparison, embryo heads carry an
essentially higher concentration of the viruses, the vaccines
prepared only from embryo heads have a correspondingly lower
content of byproducts and cause fewer side effects (German
15 Patent 3,009,064; U.S. Patent 4,255,520).

However, in the course of preparing vaccines from nerve
tissue of animals (from embryos or from embryo heads),
viruses are often damaged or fragmented when the virus-
containing tissues are homogenized with a mixer or blender.
20 This considerably reduces the activity of the vaccine
prepared from homogenates of this type and makes its purifi-
cation more difficult since large amounts of proteins
and liquids are released from the fragmented cells.

A slightly better vaccine has only been obtained by
25 multiplication of the rabies viruses by in vitro culturing
human diploid cells (HDC) (H. Koprowski, "Vaccine for man

- 3 -

1271708

- prepared in human diploid cells", Laboratory Techniques in Rabies by M.M. Kaplan and H. Koprowski, WHO Monograph Series No. 23, Chapter 28, pp. 256-60 (1973); T.J. Wiktor, Develop. Biol. Standard, Vol. 37, pp. 256-66, S. Karger, Basel 1978, "Production and control of rabies vaccines made on diploid cells"; T.J. Wiktor et al. "Development and clinical trial of rabies vaccine of tissue culture origin", Develop. Biol. Standard, Vol. 40, pp. 3-9 (1978)). The thus obtained vaccines contain human proteins as contaminants.
- 10 Such proteins, however, although producing fewer secondary reactions than do heterologous proteins, still produce some.
- A considerable disadvantage of this method is the relatively low multiplication rate of the rabies viruses in diploid fibroblast cells. This requires the use of a 10- to
- 15 25-fold greater concentration of the vaccine. Hence, this method is not efficient enough to meet world-wide demand for rabies vaccine in an economically feasible manner.
- The preparation of a rabies vaccine in duck embryo cell cultures is described in U.S. Patent 3,674,862. In this
- 20 process, however, the multiplication rate in cell cultures is limited, (U.S. Patent 3,973,000 describes a method for the enrichment of rabies viruses by density gradient centrifugation; M. Rolle and A. Mavry; Mikrobiologie, Infektions- und Seuchenlehre, Stuttgart (Microbiology, Infection and
- 25 epidemiology): 489-493 Stuttgart (1978) describe the traditional preparation of duck embryo rabies vaccine).

- 4 -

1271708

Thus, there is a pressing need for a new and highly active rabies vaccine which contains mechanically intact viruses with fully retained antigenic activity, which is straightforward to prepare and thus not too costly, and
5 free of side effects. Such vaccine would be an effective and well-tolerated vaccine which has long been sought for world-wide control of the fearsome and fatal rabies disease.

SUMMARY OF THE INVENTION

The present invention provides a process for obtaining
10 inactivated rabies viruses which are substantially myelin-free, comprising:

- (1) intracerebrally inoculating an experimental animal with whole live rabies viruses;
- (2) allowing for said viruses to multiply;
- 15 (3) comminuting nerve tissue from the animal's brain to obtain a cell suspension, said comminution being conducted in the absence of a mixer to preserve the integrity of the viruses;
- (4) separating live whole viruses from the cell
20 suspension;
- (5) delipidating the live whole viruses; and
- (6) selectively concentrating the viruses; wherein steps (1) through (4) are conducted at least once and up to
3 times.

- 5 -

1271708

In addition, this invention also provides a process for obtaining inactivated rabies viruses which are substantially myelin-free comprising:

- 5 (1) inoculating a poultry embryo egg with whole live rabies viruses;
- (2) allowing for said viruses to multiply;
- (3) comminuting the embryo from the poultry egg to obtain a cell suspension; said comminution being conducted in the absence of a mixer to preserve the integrity of the
- 10 viruses;
- (4) separating live whole viruses from the cell suspension;
- (5) delipidating the live whole viruses; and
- (6) selectively concentrating the attenuated viruses;
- 15 wherein steps (1) through (4) are performed at least once and up to 3 times.

This invention also provides a rabies vaccine comprising inactivated rabies viruses which are substantially myelin-free, said viruses being present in an amount

20 effective to elicit an immunizing response when administered to a subject. The present vaccine may be obtained by the hereinabove processes.

- 6 -

1271708

DESCRIPTION OF THE INVENTION

The present invention relates to an economic process for preparing a rabies vaccine which is of the highest quality when compared to vaccines obtained from viruses multiplied in
5 diploid human cell cultures.

In one aspect of this invention the process comprises

- (1) isolating the rabies viruses which have multiplied in animal nerve tissue or poultry embryos avoiding mechanical damage to or fragmentation of the viruses thereof;
- 10 (2) removing lipids from the resulting viruses by extraction with a water-immiscible organic solvent such as volatile paraffin hydrocarbons or halogenated hydrocarbons such as fluorinated hydrocarbons;
- (3) enriching the delipidated viruses by density
15 gradient centrifugation;
- (4) precipitating the viruses by addition of a polyethylene glycol (e.g., PEG 6000) and concentrating by centrifugation and purifying the live whole viruses.

Step (1) entails extracting the rabies virus by cautious
20 comminution (preserve cell and viral integrity) of the nerve tissue or poultry embryo heads (e.g., duck, chicken or quail), and washing the tissue fragments with a buffer, e.g., phosphate-containing buffer. This step is superior than homogenizing in a mixer or blender since foreign proteins and
25 lipids are solubilized to a lesser extent, the occurrence of

- 7 -

1271708

oxidation products of antigens, proteins and lipids is avoided, and the content of intracellular, incomplete and non-immunizing rabies antigen is diminished. The virus-containing suspension obtained by washing the tissue fragments with an aqueous buffer solution is then removed by differential centrifugation.

At least 95% of the residual protein is discarded by operations 3 and/or 4.

The viruses are then finally inactivated in a known manner, for example by addition of β -propiolactone or tri-(n-butyl)phosphate.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The individual process steps of the inventive process are performed in such a manner that a surprisingly good overall result is achieved. Harvesting viruses only from the heads of the embryos entails producing a high basic concentration of the viruses. The mild treatment of the virus-containing tissue material gives a fine paste, especially upon avoiding the homogenization thereof with a mixer or blender, provides a viral suspension which exhibits substantially no mechanically damaged or fragmented viruses with incomplete antigen content, and which, moreover, contains far fewer foreign materials such as cell debris, proteins and lipids. The remaining lipids can be removed from this viral suspension by extraction, and the proteins

- 8 -

1271708

can be far more completely removed by selective concentration
and/or precipitation of the virus than from a pasty
homogenate. The vaccine obtained by the process comprising
the sequence of steps described is improved by around 90-fold
5 compared with the conventional duck embryo rabies vaccine.

When the preparation of rabies vaccine is obtained by
passaging on nerve tissues of animals such as mice, rats,
rabbits and sheep, the viruses are multiplied in the living
animal by intracerebral inoculation of rabies viruses
10 of standardized seed strains. It must, however, be noted
that the multiplication of the viruses in living animals has
the disadvantage, compared with the multiplication of the
viruses in poultry embryos, that the nerve tissues of living
animals contain myelin. This protein is known to give rise
15 to secondary reactions when the vaccine is used, including
encephalitis.

After slaughtering the animals, which were previously
inoculated with live whole rabies viruses, their brains are
removed, comminuted in a manner which preserves the wholeness
20 of the cells and the viruses, and a vaccine is prepared from
the resulting cell suspension by the process described
hereinaabove. Owing to the avoidance of cell fragmentation
during the comminution of the virus-containing nerve tissues,
less myelin is released than during comminution with a
25 mixer. During the extraction of the lipids with an organic
solvent in a later step of the process a further part of the

- 9 -

1271708

still present myelin is removed in such a manner that the vaccine which is finally obtained causes only minimal, if any, secondary reactions, and those which are caused are still highly tolerable.

- 5 The steps of the process must be conducted avoiding the use of a mixer or blender for the homogenization of nerve tissues or of embryos or embryo heads on harvesting of the viruses. This prevents damage to and fragmentation of the viruses by comminution of the nerve tissues or of the embryos
10 or embryo heads and their contents in a manner which preserves the wholeness of the cells and the viruses, separating the complete live viruses which are capable of multiplication from the resulting cell suspension, and purifying the resulting viral suspension, delipidating by
15 extraction with a water-immiscible organic solvent and then selectively concentrating the viral preparation.

- The comminution of the nerve tissue, the embryos or embryo heads or their contents, is carried out with the aid of a meat mincer on a coarse setting, by cutting up or by
20 opening of the heads and comminuting the removed brain tissue in a manner which preserves the integrity of the cells, and therefore the viruses.

- Washing or extracting the viruses from the comminuted tissue is carried out with a buffer solution, preferably with
25 an aqueous phosphate buffer of about pH 7-8, as is known in the art. The removal of the lipids is carried out by

- 10 -

1271708

extraction with a water-immiscible solvent, such as liquid, volatile, optionally halogenated hydrocarbons. Suitable solvents are petroleum ethers such as heptanes, fluorinated and chlorinated ethanes and homologs thereof. However other solvents can also be used. The further concentration of the delipidated viral suspension can be carried out by density gradient centrifugation and/or precipitation with a polyethylene glycol, preferably with PEG 6000, as is known in the art. Suitable types of embryo poultry eggs for the multiplication of rabies viruses are in particular those from ducks, chickens and quails. In general, incubated duck eggs are preferred as the tissue for the multiplication of the viruses. The myelin-free rabies vaccine provided herein may be obtained from poultry embryo head tissue which contains rabies viruses by the process which is described above, which process fully preserves viral integrity.

The processes according to the invention results in a rabies vaccine which, compared with the vaccines obtained by processes hitherto known, exhibits a far better ratio of antigen content to protein content, contain substantially no foreign lipids, and approach in quality an ideal MDC vaccine.

1271708

DETAILED DESCRIPTION OF THE PROCESS

Now the process will be described in relation to each separate step.

Step 1:

- 5 A rabies virus strain which is suitable for the preparation of the vaccine is adapted to the intended viral host by appropriate passages on the embryonal cells of poultry eggs or in mice, rats, rabbits or sheep, among others.
- 10 Attenuated rabies viruses are, for example, inoculated into the yolk sac of fertilized poultry eggs which have undergone initial incubation and in which an embryo has started to develop. After about two weeks, the embryos are removed and their heads are harvested. The embryo heads are
- 15 comminuted in a manner which preserves cell and viral integrity in a meat mincer.
- Alternatively, the head of the embryo is cut open and the brain tissue is removed and comminuted. The multiplication of the viruses may also be undertaken in
- 20 living animals. In such case, animals which are only a few days old (mice, rats, rabbits, lambs, etc.) are usually inoculated intracerebrally with the same species-specific attenuated seed virus.
- After about 10-30 days the animals are sacrificed,
- 25 and the brains are removed by operation and comminuted in a

- 12 -

1271708

manner which preserves cell and viral integrity. The extraction of the rabies virus from the comminuted tissue is carried out by washing the tissue fragments with a phosphate-containing buffer. A suitable phosphate buffer is one comprising, e.g., 0.75% by weight of disodium hydrogen phosphate (Na_2HPO_4), 0.145% by weight of potassium dihydrogen phosphate (KH_2PO_4) and 0.48% by weight of sodium chloride in distilled water, pH 7.4. However, other buffer solutions known in the art may also be used. It is equally possible to use for the extraction, stabilizers and salt solutions which are customarily used for the preparation of viral vaccine suspensions, or even deionized water as long as the pH is in the range between 7 and 8.

The suspension containing the viral antigens is separated from the tissue by differential centrifugation at about 10,000-15,000 x g (g being the acceleration of gravity). The remaining tissue sediment can be used for further extractions, by which means a yield of about 30% of viral antigen is possible. The two or more virus-containing extracts are combined and then filtered.

Step 2:

The foreign lipids still remaining in the viral suspension are removed by extraction with a water-immiscible organic solvent, such as, e.g., with an hydrocarbon, optionally halogenated and preferably fluorinated.

- 13 -

1271708

Subsequently, the antigen extract (the viral suspension) is enriched by density gradient centrifugation in a manner known per se at 15,000-90,000 x g using a buffer and sugar solutions of various concentrations, by increasing the sugar concentration in the buffer in a manner known in the art. Alternatively, the viral suspension can be concentrated by precipitation with polyethylene glycol.

Step 3:

The density gradient centrifugation is carried out in a manner known per se at 15,000 to 19,000 x g using sugar solutions of various concentrations and buffer solutions, by increasing the sugar concentration in a buffer solution. For this purpose, the prepurified suspension is pumped at 15,000 to 90,000 x g at a flow rate of, e.g., 4 litres/h over a step gradient of an increasing concentration of sugar (usually, sucrose from 15 to 55 %) which has previously been introduced. The fractions collected from the various densities are then subjected to tests for density, the contents of lipids, nucleoproteins and glycoproteins, and sterility.

The antigen-containing fractions are pooled, tested once more, and then processed further to obtain the vaccine. Physiological saline solutions of any type, e.g., the phosphate buffer mentioned above, can be used for dilution in a manner known per se (Duck embryo rabies vaccine:

- 14 -

1271708

J.M. Hoskins, Laboratory Techniques in Rabies by M.M. Kaplan et al., WHO Geneva 1973, Chapter 27, pages 243-55; Density gradient centrifugation: J. Miltenhaus et al., J. Biol. Standard. 4:263-271 (1973); M. Majer et al., Develop. Biol. Standard. 37:267-271 (1977); and P. Atanasiu et al., Develop. Biol. Standard. 40:35-44.

Step 4:

In addition or alternative to the enrichment of the virus concentration by density gradient centrifugation, the prepurified, and usually enriched, viral suspension can be further concentrated and purified by precipitation with a polyethylene glycol, preferably free of heterologous protein. For this purpose, the pH of the viral suspension can be adjusted to about 8. After addition of a polyethylene glycol (e.g., PEG 6000) to a final concentration of 6% by weight, the suspension is stirred for at least one hour and the virus is precipitated by subsequent centrifugation at 10,000--15,000 x g. The viral sediment is then resuspended in a stabilizer composed of a solution containing lactose and physiological gelatin (E.M. Mikhailovsky et al., Ann. Inst. Pasteur 121:563-568 (1971); James McGlarry et al., Virology 40:745-746 (1970).

1271708

Step 5:

The intact live viruses capable of multiplication which are present in the resulting viral concentrate are now inactivated. Beta-propiolactone (BPL) is usually used
5 for the inactivation (G.A. LeGrippe, Annals New York Acad. of Sci. 83:578-94 (1960). However other substances are also
suitable for this purpose such as tri(n-butyl) phosphate (H. Tint et al., Symposia series in "A new tissue culture
10 rabies vaccine, inactivated and disaggregated with tri-(n-butyl) phosphate" Immunobiol. Standard. (Karger, Basel) 21:132-144; T.J. Wiktor et al., Develop. Biol. Standard. 40:3-9 (1978).

The vaccine concentrate obtained by the new process
15 differs from commercially available rabies vaccines in its high content of antigen value units per mg of nitrogen (measured using the standard MIX test in mice and the antibody binding test in the RFFIT). Preferably, the vaccine contains more than 10 antigen value units per mg of nitrogen, and still more
20 preferably more than 15 units per mg of nitrogen, but always more than 8 units per mg of nitrogen. As a rule, the same can be obtained using unborn embryos which do not as yet feel pain and in which the brain tissue, which is just in the process of development, appears to be still free of
25 myelin (M. Abdussalen et al., "The problem of anti-rabies vaccination", International conference on the application of

- 16 -

1271708

vaccine against assay viral rickettsial and bacterial diseases of man, Pan. Am. Health Org. (PAHO), Sc. pub. No. 226:54-59 (1970); and P. Fenle, "The status of existing rabies vaccines", *ibid.* pages 60-65).

5 Step 6:

The vaccine resulting after the inactivation can be dispensed into vials and can then be freeze-dried. It may be reconstituted for use by dissolution or suspension using distilled water.

- 10 As is well understood by those skilled in the art of viral purification, additional steps may be included to further purify the rabies virus.

It is possible by the process which has been described herein to prepare unlimited, or at least adequate, amounts of a valuable and innocuous rabies vaccine in an economic and relatively straightforward manner. The preparation of such quality rabies vaccine by multiplication of the viruses in human diploid cell cultures (HDC) is highly impossible as a consequence of the low efficiency of the substrate.

- 20 It is noteworthy that by an order of February 1979, the CDC has restricted the use of human diploid cell rabies vaccine to people having developed life-threatening side effects after administration of the duck embryo vaccine or who were incapable of acquiring an appropriate titer of antibodies. The reason given for this is inadequate

- 17 -

1271708

productivity of the human diploid cell cultures (See also, Morbidity and Mortality Weekly Report (MMWR) 27:333, 413 (1978))).

The rabies vaccine prepared by the process according to
5 the invention is at least equivalent to an HDC vaccine in
which the viruses have been multiplied in human diploid cell
cultures (See, Example 1 hereinbelow). No side effects have
been observed upon administration of this vaccine up to the
present time, thereby making available for medical use a
10 rabies vaccine of excellent value and effectiveness and which
has negligible side effects.

When the antigen of this invention is used to induce
immune response in a human or animal, it is administered in
an amount sufficient to elicit an immunizing response. The
15 amount of antigen may be adjusted by a clinician doing the
administration, as commonly occurs in the administration of
vaccines and other viral agents which induce immunizing
responses. Suitable vaccine unit amounts are between about
2.5 units and 10 units, preferably between about 4
20 units and 6 units. Although a single administration
induces an immune response, multiple administrations may be
carried out if desired or if so required in accordance with
schedules known per se. The route of administration can be
any of the routes generally used for rabies vaccines, such as
25 by injection subcutaneously, intramuscularly and the
like.

- 18 -

1271708

Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein for purposes of illustration only and are not intended to be limiting of the invention or any embodiment thereof, unless so specified.

EXAMPLES

Example 1 - PREPARATION OF A PURIFIED DUCK EMBRYO RABIES VACCINE

1. Preparation of the virus suspension

(a) The "Wistar rabies, PM (Pitman-Moore) SRDCS" virus strain from the Wistar Institute, Philadelphia, or another rabies virus strain suitable for the preparation of a vaccine was adapted to the embryo cells before actual use by intracerebral passage in mice and repeated passage by inoculation in duck eggs which have undergone initial incubation. The viruses used for the preparation of the vaccine are those from a passage with a particularly high titer and which have already proved to be suitable in the preparation of rabies vaccine in accordance with the method of J.M. Hoskins, "Laboratory Techniques", in Rabies by Kaplan et al., WHO, 27:243-55 Duck Embryo Vaccine (1973).

Fertilized duck eggs from healthy stocks were incubated at a temperature of $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and a humidity of 65-70%. After six days they are candled with UV light and

- 19 -

1271708

unsuitable eggs are rejected. On day 7 of incubation, the rabies virus was inoculated directly into the yolk sac of the eggs in which an embryo was developing. The incubation was continued and 10-14 days later the eggs were again candled with UV light. The eggs in which the embryos continued to develop well were opened under sterile conditions, and the embryos were removed and decapitated. The heads were stored individually under sterile conditions in the vapor phase over liquid nitrogen until the sterility tests were complete. Groups of 40-60 of the sterile heads were combined into a pool with the addition of a defined amount of a stabilizer. The sterility of each pool was again tested. In addition to the stabilizer, it was also possible to use a NaCl/phosphate buffer comprising 0.75% disodium hydrogen phosphate, 0.145% potassium dihydrogen phosphate and 0.48% sodium chloride in distilled water, or other saline solutions as are customary for the purpose of diluting vaccines, even desalinated water, as long as the pH was in the range between 7 and 8.

The rabies virus extract was obtained by comminution of the above mentioned sterile embryo heads using a meat mincer. The tissue fragments were washed twice with a phosphate-containing buffer. After centrifugation at 10,000-15,000 x g and at a temperature of 2-8°C the infectious virus was collected in the supernatant fraction. Remaining brain particles or other lipid-containing tissues

- 20 -

1271708

were removed by subsequent filtration through a gauze filter system. The remaining residues of head tissue can be extracted once more and filtered by use of the same process, by which means a higher antigen yield of about 10% is achieved. The sediment was again suspended in a phosphate-containing buffer and stirred for at least one hour at low temperature (1-4°C) before the centrifugation and filtration.

(b) A subsequent, virtually complete delipidation was carried out by mixing the resulting viral suspension with an inert liquid hydrocarbon solvent with a relatively low density, such as, for example, n-heptane. Homogenization was carried out in every case under a glass ball containing nitrogen gas. The viral suspension was pumped through a mixer system, e.g., Virtis mixer, at a constant flow rate of, e.g., 500 ml/min. At the same time, the n-heptane was pumped into the mixer system at a rate of 50 ml/min. The lipid-containing phase was removed by centrifugation at 10,000-15,000 x g. Traces of the dissolved hydrocarbon solvent were then removed from the delipidated virus extract by allowing an inert gas such as, e.g., nitrogen, to bubble through the aqueous phase and maintaining the aqueous phase under vacuum at 4°C for a period of about 15 hours.

(c) An alternative process for the hydrocarbon delipidation is as follows. Sterile embryo heads may be

- 21 -

1271708

comminuted, extracted and filtered as described under (1a).
The removal of the foreign lipids may then be carried out by
using the fluorinated hydrocarbon solvent
1,1,2-trichlorotrifluoroethane. The individual working
5 steps remain the same.

11. Concentration and further purification of the virus
suspension

(a) The prepurified rabies viral suspension prepared by
the process described above had a viral titer between 10^7 and
10 10^8 MLD₅₀/ml. This material was further purified and
concentrated by centrifugating once or twice on a linear sucrose
gradient (15-55%) at 75,000-90,000 x g. A concentration
factor of 100:1 was attained in this manner. The
glycoprotein and nucleoprotein content (before and after
15 solubilization of the virus membrane with Triton X 100,
that is to say election of the intact virions), the virus
titer, the density and the sterility of the gradient
fractions were tested. Sterile fractions with a ratio of
rabies glycoprotein to nucleoprotein which corresponds to
20 that of the purified whole virion solution, and with a very
high infectious titer (for example 10^9 - 10^{10} MLD₅₀/ml) were
combined and reserved for further processing.

(b) A further purification and concentration of the
viral suspension can be achieved by polyethylene glycol (PEG)
25 precipitation. For this purpose, PEG 6000 (Siegfried A.G.,

1271708

Zofingen, Switzerland) was dissolved in a 30% strength phosphate-containing buffer solution (pH 8.0). This stock PEG solution was sterilized in an autoclave and stored at 4°C. The viral suspension which was adjusted to a pH of 8.0 with a 10% NaOH solution was then precipitated with the stock PEG solution at a final dilution of 6%. The mixture was stirred at a temperature of 4°C for at least one hour. The rabies virus can then be sedimented by centrifugation at a speed of 10,000-15,000 x g over a period of 30 min. The removed virus was again suspended with a stabilizer to the final volume and was reserved for further processing.

III. Formulation of the viral concentrates

Pretested viral concentrates were combined and diluted with a suitable stabilizer, for example sodium phosphate buffer (pH 7.4), with a physiological sodium chloride solution, or with another stabilizer which has already been described (see, Hoskins, l.c.) to a concentration of about $10^{7.5}$ MLD₅₀/ml. Sterility and virus titer were tested again.

20 IV. Inactivation of the viruses

For the inactivation with beta-propiolactone, the final volume of the viral suspension was maintained at a temperature of 1-4°C with continuous stirring. Freshly prepared, ice-cold aqueous beta-propiolactone solution was

- 23 -

1271708

added in an amount such that a concentration of 1:4,000 was attained. After the suspension was stirred at a temperature of 4°C for 5 min, it was transferred into a second vessel and stirred for a further 40 hours; the pH and temperature were continuously monitored. A decrease in the pH was taken as a measure of BPL hydrolysis. As recorded, the pH fell from about 8.0 to about 7.4. At the end of the inactivation, thiomersal (o-(ethylmercurio)-benzoic acid) was added until the concentration of this antiseptic substance was 1:10,000.

V. Freeze-drying

The inactivated viral suspension obtained in accordance with section IV was dispensed in single doses of 1 ml into 2 ml vials, freeze-drying stoppers were placed loosely on top, and the vaccine was freeze-dried in vacuo. When the drying process was completed the stoppers were pushed in tight and the vials were closed with metal caps to assure the tightness of the vials. The vials were then stored at a temperature of -20°C.

VI. Reconstitution to give the vaccine ready for use, and use of this vaccine

Prior to its use, 1 ml of sterile distilled water was injected through the rubber stopper into each vial. The vial was then shaken cautiously, without forming a foam, until the

- 24 -

1271708

vaccine was completely dissolved. The entire content of the vial was then injected subcutaneously into the upper arm of the subject.

VII. Quality control of the final product - tests

5 The quality control procedures comprised: the determination of the antigenicity, sterility, inactivity, innocuousness and contents of nitrogen, cholesterol, NaCl, BFL residues and thiomersal.

Antigenicity:

10 Antigens were tested in accordance with standard instructions of the National Institute of Health, USA. Their ability to bind antibodies in the RFFIT test was also measured (R.J. Arko et al., Laboratory Techniques in Rabies, 3rd edition, WHO Monograph Series 23:265-267 (1973); and
15 J.S. Smith et al., Lab. techn. in Rab., 3rd edition, WHO Monograph Series 23:354 to 357 (1973).

- 25 -

1271708

Sterility:

All the final products for use were proven to be sterile.

Inactivity:

- 5 This was tested in every case on three young rabbits and ten mice, which, after intracerebral inoculation of the reconstituted vaccine, were observed for 14 days. The animals showed no signs of disease in any case.

10 Innocuousness:

- Three guinea pigs received 5 ml intraperitoneal doses of the reconstituted vaccine solution, and 3 mice received 0.5 ml i.v. doses. In no case did the animals show reactions differing from normal.

- 15 The stability of the vaccine obtained in accordance with the above description in the freeze-dried form was also tested. Efficacy (AGV-U/ml as a percentage of the initial figure (0)) was preserved after storage at the stated temperature for 3 months.

- 20 (a) Stability of the vaccine obtained in accordance with Example 1 (concentration in accordance with 2a) in the freeze-dried form. The

1271708

Activity (AGV-U/ml) as a percentage of the initial figure (0 figure) is shown in Table 1, hereinbelow.

Table 1: Activity of the Vaccine (Example 1-2a)

Batch number	0 figure AGV-U/ml	+ 37°C 1 month	+ 37°C 2 months
83 Ly III T16	6.7	100%	110%
83 Ly III T18	7.3	93%	92%

(b) Stability of the rabies vaccine obtained in accordance with Example 1 (concentration in accordance with 2b) in the freeze-dried form. The activity (AGV-U/ml) as a percentage of the initial figure (0 figure) is shown in Table 2 hereinbelow.

Table 2: Activity of the Vaccine (Example 1-2b)

Batch number	0 figure AGV-U/ml	+ 37°C 1 month	+ 37°C 2 months
83 Ly III T15	5.0	148%	98%
83 Ly III T19	8.2	107%	104%
83 Ly III T20	9.7	144%	76%
83 Ly III T21	15.3	124%	92%
83 Ly III T22	8.5	165%	105%
83 Ly III T23	13.4	105%	112%
83 Ly III T23	9.5	147%	126%

- 27 -

1271708

(c) The activity of the rabies vaccine obtained in accordance with Example 1 in a dog after s.c. inoculation is shown in Table 3 hereinbelow..

Table 3: Activity of Vaccine (Example 1, dog)

Number of inoculated animals with more than	Vaccine according to (2a), 7 dogs	Vaccine according to (2b), 8 dogs
0.5 IU	100%	100%
1	85%	88%
2	43%	75%

IU = international units of antibody content

(d) Comparative activity in humans of the rabies vaccine obtained in accordance with Example 1 and the HCD vaccine (Behring).

The activity of the new vaccine was compared with that of the HCD vaccine (Behring). Table 4 hereinbelow shows the percentage of subjects which immunologically reacted by forming antibodies after administration of one of these vaccines, in general 0.5 IU being regarded as conferring protection (inoculation on days 0, 3, 7, 14 and 28).

- 28 -

1271708

Table 4: Comparative Activity of Inventive Vaccine and HDC Vaccine

Antibody titer (RFFIT) (Day 14)	Vaccine (Ex. 1 (2b))	Vaccine (Ex. (2a))	HDC vaccine (Bähring-Werke)
	3 consecu- tive batches 54 subjects	2 consecu- tive batches 15 subjects	AGV: 6.3 IU 20 subjects
0.5 IU	100%	100%	100%
2	98%	100%	100%
5	80%	80%	80%
10	45%	60%	50%
15	28%	47%	30%

IU = international units of antibody content.

Results

The rabies vaccine prepared by the process according to the invention proved in the clinical trial to be of at least equal quality to an HDC vaccine, wherein the viruses had been multiplied in human diploid cell cultures (HDC).

Example 2 - Preparation Of A Purified Duck Embryo Vaccine.

Rabies viruses were multiplied in duck eggs which have undergone initial incubation as described in Example 1. The viruses were separated from the embryo heads by cutting the heads open, removing the brain tissue and comminuting in a

1271708

manner preserving cell and viral integrity, and were harvested by resuspension in a phosphate buffer solution.

The viral suspension was further processed to give a vaccine as in Example 1.

5 Example 3 - Purified Duck Embryo Vaccine.

A highly concentrated viral suspension was prepared in accordance with Example 1 and was inactivated by treatment with tri-(n-butyl) phosphate. After inactivation, the concentrate was freeze-dried.

10 Example 4 - Preparation Of Purified Chicken Embryo Vaccine.

Chicken eggs are incubated at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under a humidity of 60-75% for 7 days. On day 7 of the incubation, the inoculum virus was directly inoculated into the yolk sac of the embryos undergoing development in the eggs. The incubation continued. Seven days later, the eggs were opened and the embryos were removed and dismembered. The heads, the spinal cord and the trunks were processed separately. They were comminuted in a manner such that they provided a 10% strength tissue suspension (that is to say a suspension of 10% by weight of embryo tissue). The viral concentration was titrated using Antibody binding test in the RFFIT (rapid Fluorescent focus Inhibition Test). Three series of tests were carried out; the results of which are shown in the table hereinbelow.

- 30 -

1271708

Table 5: Preparation of Chicken Embryo Vaccine

	Viral concentration (ID 50/ml)
Head	7.25 / 7.4 & 7.8
Spinal cord	6.55 & 7.8
Trunk	6.0 / 6.6 & 6.8

ID = 50/ml = virus titer x log 10 for the minimum concentration for infection of 50% of the tissue cultures.

It was found that the tissue of the central nervous system (CNS) contained about 10 times as much viruses as the trunk without the CNS.

On the basis of these results, the preparation of the chicken embryo vaccine by the method described in Example 1 was carried out only with the embryo heads. In the present case, the rabies viruses which had been adapted to chicken cells was multiplied in partially incubated chicken eggs, e.g., by the method of H. Koprowsky (Laboratory technique in Rabies by M.M. Kaplan et al., WHO Geneva, Chapter 26, pages 235-242).

The eggs were inoculated on day 7 of incubation and incubation was continued the next day. Seven to nine days after inoculation of the virus into the yolk sac, the heads

- 31 -

1271708

of the chicken embryos were removed and processed in accordance with the method described in Example 1. This entailed the final concentration of the viruses being carried out by precipitation with polyethylene glycol. The vaccine
5 prepared in this manner was subjected to the quality control tests described under I through VII. This vaccine also proved to be fully active in humans.

Example 5 - Preparation Of Quail Embryo Vaccine.

In a manner analogous to that described in Example 1,
10 attenuated rabies viruses were multiplied in quail eggs which had undergone initial incubation, and were harvested and processed to obtain the vaccine. The resulting vaccine proved to be fully active in animal experiments.

Example 6 - Preparation Of A Rabies Vaccine From Viruses

15 Multiplied In Mice.

Five-day old mice were inoculated intracerebrally with attenuated seed rabies viruses. After ten days, the mice which were still alive were sacrificed. The brains of the animals were removed and comminuted in a manner which
20 preserved cell and viral integrity. The cell suspension was processed to give a vaccine by the process of Example 1.

1271708

Example 7 - Preparation Of A Rabies Vaccine From Viruses
Multiplied In Rats.

3-4 day old rats were inoculated intracerebrally with
attenuated seed rabies viruses. After 12 days, the rats
5 which were still surviving were sacrificed. The brains of the
animals were processed to give the vaccine in analogy to
Example 6.

Example 8 - Rabies Vaccine From Viruses Multiplied
In Rabbits.

10 Six day old rabbits were inoculated intracerebrally with
attenuated seed viruses. After 15 days the rabbits were
sacrificed. The brains of the animals were processed to give
a vaccine in analogy to Example 6.

Example 9 - Vaccine From Viruses Multiplied In Lambs.

15 8-10 day old sheep were inoculated intracerebrally with
attenuated seed rabies viruses. After 30 days, the lambs
were sacrificed. Their brains were removed and processed to
give a vaccine in analogy to Example 6.

1271708

Having now fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit of the scope of the invention as set forth herein.

- 34 -

1271708

WE CLAIM:

1. A process for obtaining inactivated rabies viruses, comprising:
 - (1) intracerebrally inoculating an experimental animal with whole live rabies viruses;
 - (2) allowing for said viruses to multiply;
 - (3) comminuting nerve tissue from the animal's brain to obtain a cell suspension, said comminution being conducted in the absence of a mixer to preserve the integrity of the viruses;
 - (4) separating live whole viruses from the cell suspension;
 - (5) delipidating the live whole viruses; and
 - (6) selectively concentrating the viruses; wherein steps (1) through (4) are conducted at least once and up to 3 times; said viruses being substantially myelin-free.
2. The process of claim 1 wherein the viruses are selectively concentrated by density gradient centrifugation or precipitation with a polyethylene glycol.
3. The process of claim 1 further comprising inactivating the viruses.

- 35 -

B

1271708

4. The process of claim 1, wherein the viruses are separated from the cell suspension by washing and suspending thereof in a physiological buffer solution having a pH about 7 to 8.

5. The process of claim 1 wherein the viruses are delipidated by adding a water-immiscible liquid organic solvent.

6. The process of claim 1, wherein the solvent is a hydrocarbon selected from the group consisting of water-immiscible liquid hydrocarbons or halogenated hydrocarbons.

7. The process of claim 6 wherein the delipidating hydrocarbon is an halogenated hydrocarbon.

8. The process of claim 6 wherein the hydrocarbon is selected from the group consisting of a petroleum ether, fluorinated or chlorinated ethane and homologues thereof.

9. The process of claim 8 wherein the petroleum ether is heptanes.

- 36 -

1271708

10. The process of claim 1 wherein the animal is selected from the group consisting of mice, rats, rabbits and sheep.

11. The process of claim 1 further comprising precipitation and concentrating the viruses.

12. The process of claim 3 wherein the viruses are inactivated by adding a virus-inactivating amount of β -propiolactone or tri-(n-butyl)phosphate.

13. The process of claim 1 further comprising placing said viruses in a sterile vial and freeze-drying thereof.

14. A rabies vaccine comprising inactivated rabies viruses which are substantially myelin-free in an amount effective to elicit an immunologizing response when administered to a subject.

15. The rabies vaccine of claim 14 having at least 10 antigen value units per mg of nitrogen.

16. The rabies vaccine of claim 14 in dosage unit form.

- 37 -

1271708

17. A rabies vaccine comprising attenuated rabies viruses obtained by the process of claim 1, said vaccine being substantially myelin-free and said viruses being present in an amount effective to elicit an immunizing response when administered to a subject.

18. The rabies vaccine of claim 17 having at least 10 antigen value units per mg of nitrogen.

19. The rabies vaccine of claim 14 in dosage unit form.

20. A process for obtaining attenuated rabies viruses comprising

inoculating a poultry embryo egg with whole live rabies viruses;

allowing for said viruses to multiply;

comminuting the embryo from the poultry egg to obtain a cell suspension; said comminution being conducted in the absence of a mixer to preserve the integrity of the viruses;

separating live whole viruses from the cell suspension;

delipidating the live whole viruses; and

selectively concentrating the viruses; wherein steps (1) through (4) are performed at least once and up to 3 times; said viruses being substantially myelin-free.

1271708

21. The process of claim 20 further comprising inoculating the viruses.

22. The process of claim 20 further comprising conducting the following steps at least once prior to inoculating the poultry embryo

intracerebrally inoculating an experimental animal with live whole rabies viruses;

allowing for the viruses to multiply; and

separating live whole viruses from brain tissue.

23. The process of claim 20, wherein the attenuated viruses are selectively concentrated by density gradient centrifugation or precipitation with a polyethylene glycol.

24. The process of claim 20, wherein the viruses are separated from the cell suspension by washing and suspending thereof in a physiological buffer solution pH about 7 to 8.

25. The process of claim 20 wherein the viruses are delipidated by adding a liquid water-immiscible organic solvent.

26. The process of claim 25, wherein the solvent is a hydrocarbon selected from the group consisting of liquid, volatile hydrocarbons or halogenated hydrocarbons.

- 35 -

1271708

27. The process of claim 26 wherein the delipidating hydrocarbon is an halogenated hydrocarbon.

28. The process of claim 26 wherein the hydrocarbon is selected from the group consisting of a petroleum ether, fluorinated or chlorinated ethane and homologues thereof.

29. The process of claim 28 wherein the petroleum ether is heptane.

30. The process of claim 22 wherein the experimental animal is selected from the group consisting of mice, rats, rabbits and sheep.

31. The process of claim 21 wherein the viruses are inactivated by adding a virus-inactivating amount of p-propionolactone or tri-(n-butyl)phosphate.

32. The process of claim 20 further comprising precipitating and concentrating the viruses.

33. The process of claim 20 wherein the embryonic poultry eggs are selected from the group consisting of embryonic duck, chicken and quail eggs.

- 40 -

1271708

34. The process of claim 20 further comprising placing said viruses in in sterile vial and freeze-drying thereof.

35. The process of claim 20 wherein the cell suspension containing the viruses is obtained by comminuting the beads of the embryos.

36. A rabies vaccine comprising inactivated rabies viruses obtained by the process of claim 20 said vaccine being substantially myelin-free and said viruses being present in an amount effective to elicit an immunizing response when administered to a subject.

37. The rabies vaccine of claim 36 containing at least 10 antigen value units per mg of nitrogen.

38. The rabies vaccine of claim 36 in dosage unit form.

⑬ BUNDESREPUBLIK
DEUTSCHLAND



DEUTSCHES
PATENTAMT

⑫ Offenlegungsschrift
⑪ DE 33 10263 A 1

⑴ Aktenzeichen: P 33 10 263.5
⑵ Anmeldetag: 22. 3. 83
⑶ Offenlegungstag: 27. 9. 84

⑮ Int. Cl. 3:
B 01 D 13/00
C 02 F 1/44
A 61 M 1/03

DE 33 10263 A 1

⑴ Anmelder:

Fresenius AG, 6380 Bad Homburg, DE

⑵ Erfinder:

Brunner, Gorig, Prof. Dr.med., 3000 Hannover, DE;
Krick, Gerd, Dr.-Ing., 6380 Bad Homburg, DE;
Mathieu, Bernd, Dr., 6683 Spiesen, DE

⑸ Verfahren zur Entfernung von lipophilen Stoffen aus wässrigen Lösungen sowie Vorrichtung zur Durchführung des Verfahrens

Verfahren zum Entfernen von lipophilen Stoffen aus wässrigen Lösungen, insbesondere aus biologischen Flüssigkeiten, bei dem die zu reinigende Flüssigkeit durch eine polymere Membran von der Reinigungsflüssigkeit getrennt ist und als Reinigungsflüssigkeit ein lipophiles Lösungsmittel eingesetzt wird. Das Verfahren eignet sich insbesondere zur Abtrennung von lipophilen Schadstoffen aus dem Blut, die schwere komatöse Zustände verursachen.

FRESENIUS AG

6380 Bad Homburg vdH

PATENTANWÄLTE

R.-A. KUHNEN*, DIPL.-ING.

W. LUDERSCHMIDT**, DR., DIPL.-CHEM.

P.-A. WACKER*, DIPL.-ING., DIPL.-WIRTSCH.-ING.

11 FR 0456 4/kub

Patentansprüche

1. Verfahren zur Entfernung von lipophilen Stoffen aus wässrigen Lösungen, insbesondere aus biologischen Flüssigkeiten, bei dem die zu reinigende Lösung und die Reinigungsflüssigkeit durch eine Membran getrennt sind und an dieser vorbeigeführt werden, d a -
5 d u r c h g e k e n n z e i c h n e t , daß man als Reinigungsflüssigkeit ein lipophiles Lösungsmittel einsetzt.
- 10 2. Verfahren nach Anspruch 1, d a d u r c h g e - k e n n z e i c h n e t , daß man als Reinigungsflüssigkeit eine Flüssigkeit einsetzt, die die abzutrennenden Stoffe besser löst als die wässrige Lösung.
- 15 3. Verfahren nach Anspruch 1 oder 2, d a d u r c h g e k e n n z e i c h n e t , daß man eine pharmakologisch unbedenkliche Reinigungsflüssigkeit einsetzt.
- 20 4. Verfahren nach einem der Ansprüche 1 - 3, d a - d u r c h g e k e n n z e i c h n e t , daß man als Reinigungsflüssigkeit eine in Wasser im wesentlichen

BÜRO 6370 OBERURSEL**
LINDENSTRASSE 10
TEL 06171/56049
TELEX 4186343 real d

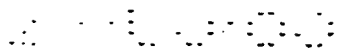
BÜRO 8260 FREISING*
SCHNEGGSTRASSE 3-5
TEL 08161/62091
TELEX 526547 pawo d

ZWEIGBÜRO 8390 PASSAU
LUDWIGSTRASSE 2
TEL 0851/36616

- 1 nicht lösliche Flüssigkeit einsetzt.
- 5 5. Verfahren nach einem der Ansprüche 1 - 4, da -
durch gekennzeichnet, daß man als
Reinigungsflüssigkeit hydrophobe organische Stoffe,
höherkettige Kohlenwasserstoffe, Paraffine, Isopara-
fine, halogenierte Kohlenwasserstoffe, Ether, höher
oxigenierte Kohlenwasserstoffe, Siliconöle, Öle tie-
rischen und pflanzlichen Ursprungs, Naphtene und/oder
10 Aromaten mit einem Molekulargewicht bis 1000 einsetzt.
- 15 6. Verfahren nach Anspruch 5, da durch ge-
kennzeichnet, daß man stark raffinierte
Mineralöle, Öle pflanzlichen und/oder tierischen Ur-
sprungs, die stark hydriert sind, dimethylierte Sili-
cone und/oder perhalogenierte Kohlenwasserstoffe ein-
setzt.
- 20 7. Verfahren nach Anspruch 5 oder 6, da durch
gekennzeichnet, daß man als Reinigungs-
flüssigkeit Baumwollsaatöl, Leinöl, Olivenöl, Rüböl,
Sojabohnenöl, Spermöl und/oder Paraffinöl einsetzt.
- 25 8. Verfahren nach Anspruch 7, da durch ge-
kennzeichnet, daß die Reinigungsflüssig-
keit in gesättigter Form vorliegt.
- 30 9. Verfahren nach einem der Ansprüche 1 - 8, da -
durch gekennzeichnet, daß die
Reinigungsflüssigkeiten eine Viskosität von 0,1 - 150,
insbesondere 10 - 80 cSt aufweisen.
- 35 10. Verfahren nach einem der Ansprüche 1 - 9, da -
durch gekennzeichnet, daß man der
Reinigungsflüssigkeit die Verunreinigungen abfangende
Mittel zusetzt.

- 1 11. Verfahren nach Anspruch 10, d a d u r c h g e -
k e n n z e i c h n e t , daß man als Ammoniak abfan-
g e n d e M i t t e l V e r b i n d u n g e n m i t e i n e r o d e r m e h r e r e n C a r -
b o x y l g r u p p e n e i n s e t z t .
- 5 12. Verfahren nach Anspruch 10 oder 11, d a d u r c h
g e k e n n z e i c h n e t , daß man als Ammoniak
a b f a n g e n d e M i t t e l h ö h e r e F e t t s ä u r e n o d e r D i c a r b o n s ä u -
r e n e i n s e t z t , d i e g g f . m i t e i n e r C a r b o x y l g r u p p e m i t
10 G l y c e r i n v e r e s t e r t s i n d .
13. Verfahren nach Anspruch 12, d a d u r c h g e -
k e n n z e i c h n e t , daß man als Ammoniak ab-
f a n g e n d e M i t t e l G l y c e r i n b e r n s t e i n s ä u r e e s t e r , O x a l -
15 e s s i g s ä u r e u n d / o d e r Z i t r o n e n s ä u r e e i n s e t z t .
14. Verfahren nach einem der Ansprüche 1 - 13, d a -
d u r c h g e k e n n z e i c h n e t , daß die
p o l y m e r e M e m b r a n v o n d e r z u r e i n i g e n d e n w ä s s r i g e n
20 L ö s u n g o d e r d e r R e i n i g u n g s f l ü s s i g k e i t b e n e t z t w i r d ,
w o b e i d i e P o r e n d e r M e m b r a n u n d g g f . d i e d e r a n d e r e n
F l ü s s i g k e i t z u g e w a n d t e F l ä c h e d e r M e m b r a n v o n d e r b e -
n e t z e n d e n F l ü s s i g k e i t b e n e t z t w e r d e n .
- 25 15. Verfahren nach Anspruch 1 oder 14, d a d u r c h
g e k e n n z e i c h n e t , daß man als Polymeri-
s a t e f ü r d i e M e m b r a n r e g e n e r i e r t e C e l l u l o s e , C e l l u -
l o s e a c e t a t , P o l y v i n y l a l k o h o l , P o l y a c r y l s ä u r e s o w i e
d e r e n E s t e r , P o l y a c r y l s ä u r e n i t r i l , P o l y (a r o m a t i s c h e) -
30 a m i d e , P o l y c a r b o n a t , P o l y s u l f o n e , P o l y e t h e r , P o l y -
e t h y l e n , P o l y p r o p y l e n , P o l y b u t e n e , P o l y u r e t h a n , P o l y -
i s o b u t y l e n , P o l y s t y r o l , P o l y v i n y l e t h e r , P o l y v i n y l -
e s t e r o d e r P T F E e i n s e t z t .
- 35 16. Verfahren nach Anspruch 1, 14 oder 15, d a d u r c h
g e k e n n z e i c h n e t , daß die polymere Mem-
b r a n e i n e D i c k e v o n 1 - 5 0 0 , v o r z u g s w e i s e 5 - 3 0 0 ,
i n s b e s o n d e r e 1 0 - 1 0 0 μ m a u f w e i s t .

- 1 17. Verfahren nach Anspruch 1 oder 15 - 16, d a -
d u r c h g e k e n n z e i c h n e t , daß der
mittlere Porendurchmesser der polymeren Membran 50 Å -
10 µm, vorzugsweise 0,01 - 1 µm, insbesondere 0,05 -
5 0,5 µm beträgt.
18. Vorrichtung zur Durchführung des Verfahrens nach An-
spruch 1, g e k e n n z e i c h n e t d u r c h
einen Behälter (12, 46), der durch wenigstens eine
10 polymere Membran (18, 48) in einer erste Behälterhäf-
te (14, 50) und eine zweite Behälterhälfte (16, 52)
geteilt ist, wobei beide Behälterhälften (14, 16, 50,
52) je eine Zulaufleitung (20, 24, 56, 64) und eine
Ablaufleitung (22, 26, 60, 68) aufweisen und die erste
15 Behälterhälfte (14, 50) die zu reinigende wässrige
Lösung (30) aufweist und die zweite Behälterhälfte
(16, 52) mit der Reinigungsflüssigkeit (38) beauf-
schlagt ist, die ein lipophiles Lösungsmittel dar-
stellt.
20
19. Vorrichtung nach Anspruch 18, d a d u r c h g e -
k e n n z e i c h n e t , daß die zweite Behälterhäf-
te (16, 52) mit einem Reservoir (66) zum Einspeisen
der Reinigungsflüssigkeit verbunden ist.
25
20. Vorrichtung nach Anspruch 18 oder 19, d a d u r c h
g e k e n n z e i c h n e t , daß die zweite Behälter-
hälfte (16, 52) mit einem Filter (78) zum Reinigen der
Reinigungsflüssigkeit verbunden ist.
30
21. Vorrichtung nach einem der Ansprüche 18 - 21, d a -
d u r c h g e k e n n z e i c h n e t , daß in der
Leitung (64) eine Einrichtung (72) zur Erzeugung eines
Druckgefälles angeordnet ist.
35
22. Vorrichtung nach Anspruch 21, d a d u r c h g e -
k e n n z e i c h n e t , daß die Einrichtung (72)



3310263

-4-

- 1 17. Verfahren nach Anspruch 1 oder 15 - 16, d a -
d u r c h g e k e n n z e i c h n e t , daß der
mittlere Porendurchmesser der polymeren Membran 50 Å -
10 µm, vorzugsweise 0,01 - 1 µm, insbesondere 0,05 -
5 0,5 µm beträgt.
18. Vorrichtung zur Durchführung des Verfahrens nach An-
spruch 1, g e k e n n z e i c h n e t d u r c h
einen Behälter (12, 46), der durch wenigstens eine
10 polymere Membran (18, 48) in einer erste Behälterhäf-
te (14, 50) und eine zweite Behälterhälfte (16, 52)
geteilt ist, wobei beide Behälterhälften (14, 16, 50,
52) je eine Zulaufleitung (20, 24, 56, 64) und eine
Ablaufleitung (22, 26, 60, 68) aufweisen und die erste
15 Behälterhälfte (14, 50) die zu reinigende wässrige
Lösung (30) aufweist und die zweite Behälterhälfte
(16, 52) mit der Reinigungsflüssigkeit (38) beauf-
schlagt ist, die ein lipophiles Lösungsmittel dar-
stellt.
20
19. Vorrichtung nach Anspruch 18, d a d u r c h g e -
k e n n z e i c h n e t , daß die zweite Behälterhäf-
te (16, 52) mit einem Reservoir (66) zum Einspeisen
der Reinigungsflüssigkeit verbunden ist.
25
20. Vorrichtung nach Anspruch 18 oder 19, d a d u r c h
g e k e n n z e i c h n e t , daß die zweite Behälter-
hälfte (16, 52) mit einem Filter (78) zum Reinigen der
Reinigungsflüssigkeit verbunden ist.
30
21. Vorrichtung nach einem der Ansprüche 18 - 21, d a -
d u r c h g e k e n n z e i c h n e t , daß in der
Leitung (64) eine Einrichtung (72) zur Erzeugung eines
Druckgefälles angeordnet ist.
35
22. Vorrichtung nach Anspruch 21, d a d u r c h g e -
k e n n z e i c h n e t , daß die Einrichtung (72)

22.1.83

3310263

-5-

1 über eine Leitung (76) mit einem Drucksensor (74)
verbunden und hierdurch steuerbar ist.

5

10

15

20

25

30

35

6

FRESENIUS AG

6380 Bad Homburg vdH

PATENTANWÄLTE

R.-A. KUHNEN*, DIPL.-ING.

W. LUDERSCHMIDT**, DR., DIPL.-CHEM.

P.-A. WACKER*, DIPL.-ING., DIPL.-WIRTSCH.-ING.

11 FR 0456 4/kub

Verfahren zur Entfernung von lipophilen Stoffen aus wässrigen Lösungen sowie Vorrichtung zur Durchführung des Verfahrens

- 5 Die Erfindung betrifft ein Verfahren zur Entfernung von lipophilen Stoffen aus wässrigen Lösungen, insbesondere aus biologischen Flüssigkeiten, bei dem die zu reinigende Lösung und die Reinigungsflüssigkeit durch eine Membran getrennt sind und an dieser vorbeigeführt werden, und eine Vorrichtung zur Durchführung des Verfahrens. Sie betrifft insbesondere ein Verfahren zur Entfernung von lipophilen, in Körperflüssigkeiten gelösten Schadstoffen, das extrakorporal durchgeführt werden kann.
- 10
- 15 Zahlreiche, für den menschlichen Organismus toxische Stoffe sind lipophiler Natur und können daher im wesentlichen nicht über die Niere ausgeschieden werden, sondern müssen in der Leber metabolisiert werden. Dabei werden sie häufig in ein wasserlösliches Produkt umgewandelt, das anschließend über die Niere ausgeschieden werden kann.
- 20

Dieser Metabolismus fällt jedoch aus, wenn es zu einem akuten Leberversagen kommt, beispielsweise durch eine

1 Erkrankung der Leber oder eine Arzneimittelüberdosis.
Durch das Leberversagen treten hohe Spiegel endogener
Toxine auf, die wiederum cerebrale Funktionen hemmen,
komatöse Zustände verursachen und überdies die Entgiftungs-
5 funktion der noch intakten Leberzellen hemmen. Der sich
hierdurch ständig hochschaukelnde Prozeß führt letztlich
zum Tod des Patienten.

In der Leber werden lipophile Toxine, beispielsweise
10 Phenole, Mercaptane und Fettsäuren, durch chemische Um-
wandlung (Hydroxilierung und Konjugierung) enzymatisch
in den wasserlöslichen Zustand überführt. Im überwiegen-
den Maß werden diese Stoffe an die Glucuronsäure mit Hil-
fe von Uridindiphosphoglucuronyltransferase (UDPGT) in
15 Form der Glucuronide gekoppelt, die wasserlöslich sind
und über die Niere ausgeschieden werden können.

Es wurden zahlreiche Versuche unternommen, diese enzyma-
tische Umwandlung zur Entfernung der Toxine nutzbar zu
20 machen. Der Einsatz von Leberhomogenaten, Gewebsscheiben
oder von ganzen Tierlebern führte nicht zu dem gewünsch-
ten Erfolg, da diese entweder schnell ihre Funktion ver-
loren oder den Toxinaustausch, wenn überhaupt, nur sehr
verzögert zuließen.

25 Man schlug daher den Einsatz von Adsorbenzien, insbeson-
dere von Aktivkohle vor, also den vermehrten Einsatz der
Hämoperfusion (vgl. Brunner u. Schmidt, Artificial Liver
Support, Springer-Verlag, Berlin, 1981, S.46 ff). Bei
30 diesem Verfahren, das hochgradig unspezifisch ist, wer-
den nicht nur Toxine, sondern auch eine außergewöhnlich
hohe Zahl von lebenswichtigen Substanzen aus dem Blut
entfernt. So sinkt beispielsweise der Spiegel der im
Blut befindlichen Hormone nahezu auf Null ab, so daß die
35 Schäden einer solchen Behandlung größer sind als ihr
Nutzen.

Erkrankung der Leber oder eine Arzneimittelüberdosis.
Durch das Leberversagen treten hohe Spiegel endogener
Toxine auf, die wiederum cerebrale Funktionen hemmen,
komatöse Zustände verursachen und überdies die Entgiftungs-
funktion der noch intakten Leberzellen hemmen. Der sich
hierdurch ständig hochschaukelnde Prozeß führt letztlich
zum Tod des Patienten.

In der Leber werden lipophile Toxine, beispielsweise
Phenole, Merkaptane und Fettsäuren, durch chemische Um-
wandlung (Hydroxilierung und Konjugierung) enzymatisch
in den wasserlöslichen Zustand überführt. Im Überwiegen-
den Maß werden diese Stoffe an die Glucuronsäure mit Hil-
fe von Uridindiphosphoglucuronyltransferase (UDPGT) in
Form der Glucuronide gekoppelt, die wasserlöslich sind
und über die Niere ausgeschieden werden können.

Es wurden zahlreiche Versuche unternommen, diese enzyma-
tische Umwandlung zur Entfernung der Toxine nutzbar zu
machen. Der Einsatz von Leberhomogenaten, Gewebsscheiben
oder von ganzen Tierlebern führte nicht zu dem gewünsch-
ten Erfolg, da diese entweder schnell ihre Funktion ver-
loren oder den Toxinaustausch, wenn überhaupt, nur sehr
verzögert zuließen.

Man schlug daher den Einsatz von Adsorbenzien, insbeson-
dere von Aktivkohle vor, also den vermehrten Einsatz der
Hämoperfusion (vgl. Brunner u. Schmidt, Artificial Liver
Support, Springer-Verlag, Berlin, 1981, S.46 ff). Bei
diesem Verfahren, das hochgradig unspezifisch ist, wer-
den nicht nur Toxine, sondern auch eine außergewöhnlich
hohe Zahl von lebenswichtigen Substanzen aus dem Blut
entfernt. So sinkt beispielsweise der Spiegel der im
Blut befindlichen Hormone nahezu auf Null ab, so daß die
Schäden einer solchen Behandlung größer sind als ihr
Nutzen.

- 1 Ein Verfahren der eingangs erwähnten Art stellt die Hämodyalyse dar, bei der die Körperflüssigkeit Blut an der
einen Seite einer Membran vorbeigeführt wird, deren andere
Seite von einer wässrigen Dialyselösung umspült wird.
5 Infolge des Konzentrationsunterschieds zwischen diesen
beiden, durch die Membran getrennten wässrigen Flüssigkeiten
diffundieren die zu entfernenden wasserlöslichen
Stoffwechselprodukte, beispielsweise Harnstoff u.dgl.
durch die Membran und werden von der wässrigen Dialyse-
10 lösung abtransportiert. Da auf beiden Seiten wässrige
Flüssigkeiten vorliegen, können im Blut solubilisierte,
lipophile Substanzen in aller Regel nicht durch die Membran
in die Dialyselösung diffundieren, die im wesentlichen
nur Elektrolytsalze aufweist und somit keine solubilisierenden
15 Eigenschaften besitzt.

- Auch mit der Hamofiltration kann dieses Problem nicht gelöst
werden, da an der Membran lediglich Wasser abgepreßt wird,
die nur wasserlösliche Bestandteile mit sich führt.
20 Es bleiben also die lipophilen Bestandteile im Blut zurück,
so daß auch hierdurch keine Abtrennung erfolgen kann.

- Es wurden daher Versuche mit einem Flüssigmembranenzym-
25 reaktor (vgl. vorstehende Monographie, S. 219) unternommen,
um mit der Flüssigmembrantechnik lipophile Substanzen,
beispielsweise Lebertoxine, zu entfernen. Dabei wird
durch spezielle Verfahrensweisen eine Flüssigmembran
zwischen der zu reinigenden Lösung und der Reinigungs-
30 lösung angeordnet, üblicherweise in Form einer Emulsion,
deren Tröpfchen die Reinigungsflüssigkeit eingeschlossen
enthält, wobei die Tropfenoberfläche durch die Flüssigmembran
gebildet wird. Diese Flüssigmembran besteht üblicherweise
aus einem nicht in Wasser löslichen, die
35 lipophilen Stoffe jedoch gut lösenden Lösungsmittel,
beispielsweise unpolaren Flüssigkeiten, wie Paraffin u.
dgl. Derartige Flüssigmembranen und Verfahren zu ihrer



CULLEN & CO.

— Patent & Trade Mark Attorneys —

3310263

- 1 Ein Verfahren der eingangs erwähnten Art stellt die Hämodyalyse dar, bei der die Körperflüssigkeit Blut an der einen Seite einer Membran vorbeigeführt wird, deren andere Seite von einer wässrigen Dialyselösung umspült wird.
- 5 Infolge des Konzentrationsunterschieds zwischen diesen beiden, durch die Membran getrennten wässrigen Flüssigkeiten diffundieren die zu entfernenden wasserlöslichen Stoffwechselprodukte, beispielsweise Harnstoff u.dgl. durch die Membran und werden von der wässrigen Dialyselösung abtransportiert. Da auf beiden Seiten wässrige
- 10 Flüssigkeiten vorliegen, können im Blut solubilisierete, lipophile Substanzen in aller Regel nicht durch die Membran in die Dialyselösung diffundieren, die im wesentlichen nur Elektrolytsalze aufweist und somit keine solubilisierenden Eigenschaften besitzt.
- 15

- Auch mit der Hämofiltration kann dieses Problem nicht gelöst werden, da an der Membran lediglich Wasser abgepreßt wird, die nur wasserlösliche Bestandteile mit sich führt.
- 20 Es bleiben also die lipophilen Bestandteile im Blut zurück, so daß auch hierdurch keine Abtrennung erfolgen kann.

- Es wurden daher Versuche mit einem Flüssigmembranenzymreaktor (vgl. vorstehende Monographie, S. 219) unternommen, um mit der Flüssigmembrantechnik lipophile Substanzen, beispielsweise Lebertoxine, zu entfernen. Dabei wird durch spezielle Verfahrensweisen eine Flüssigmembran zwischen der zu reinigenden Lösung und der Reinigungslösung angeordnet, üblicherweise in Form einer Emulsion, deren Tröpfchen die Reinigungsflüssigkeit eingeschlossen enthält, wobei die Tropfenoberfläche durch die Flüssigmembran gebildet wird. Diese Flüssigmembran besteht üblicherweise aus einem nicht in Wasser löslichen, die
- 30 lipophilen Stoffe jedoch gut lösenden Lösungsmittel, beispielsweise unpolaren Flüssigkeiten, wie Paraffin u. dgl. Derartige Flüssigmembranen und Verfahren zu ihrer
- 35

Correspondence
GPO Box 1074
Brisbane QLD 4001
Australia

Offices
Brisbane
Gold Coast

ABN 16 251 059 175

Brisbane Office
Level 26, MLC Building
239 George Street, Brisbane
QLD 4000 Australia

Telephone +61 7 3011 5555

+61 7 3221 8761

Facsimile +61 7 3229 3384

+61 7 3229 6598

Email mail@cullens.com.au

Web Site www.cullens.com.au

HELMUT A. EICHBERGER *
B.L. Esq. Grad.Dip.Law (IPA)

CLAUDE ANESE **

B.L. Hon. M.B.A. Dip.Law (IPA)

~~RONALD A. HALDWAY *~~

M.S. Hon. Dip.Law (IPA) Dip.Law (IPA)

IAN de JONGE *

B.L. Hon. PhD Dip.Law (IPA) Dip.Law (IPA)

KENNETH G. FINNEY *

B.L. Hon. PhD Grad.Dip.Law (IPA)

ALISON M. McMILLAN *
B.L. Hon. M.S. PhD Grad.Dip.Law (IPA)

ELINA MCCUTCHEON *

B.L. Hon. Dip.Law (IPA)

BARRY JAMES
Assoc. NZIQA NZIPA

WENDY DEAR

M.B. LL.B. Hon. LL.B.

GINT MIENS
B.L. Hon. PhD
DAVID MORGAN
B.L. Hon. Dip.Law
IRENE ELLUL
B.L. Practice Manager



3310263

CULLEN & CO.

— Patent & Trade Mark Attorneys —

1 Herstellung sind beispielsweise in den deutschen Patent-
schriften 16 19 867, 22 22 067, 25 18 742, 21 48 098,
24 34 550 sowie den US-PSen 34 10 794, 37 79 907 u.dgl.
beschrieben.

5

Im vorstehenden Enzymreaktor wird eine wässrige Lösung,
die die abzutrennende lipophile Substanz enthält, mit
einer Emulsion vermischt, die, wie vorstehend erläutert,
aus einer Vielzahl von Tröpfchen besteht, deren Oberflä-
10 che die Flüssigmembran aufweist. Als Reinigungslösung
enthalten diese Tröpfchen beispielsweise eine Enzymlö-
sung, die die lipophilen Substanzen in eine wasserlös-
liche Form überführen kann. Legt man beispielsweise Phe-
nol oder Naphtol in flüssiger Lösung vor und vermischt
15 diese Lösung mit dieser Emulsion, so stellt man fest,
daß das lipophile Phenol die lipophile Flüssigmembran-
schicht durchdringt, von der Enzymphase aufgenommen und
in dieser durch entsprechende enzymatische Umwandlung in
ein hydrophiles Reaktionsprodukt umgewandelt wird, das
20 nicht mehr durch die hydrophobe Membran rückdiffundieren
kann. Somit kann eines der schädlichsten Toxine aus dem
System durch Extraktion mit Hilfe einer Flüssigmembran
entfernt werden.

25

Obwohl die Extraktion mit der Flüssigmembrantechnik zu-
nächst als besonders vorteilhaft erscheint, weist sie
den Nachteil auf, daß die eingesetzten Emulsionen na-
türlich von dem zu reinigenden System abgetrennt werden
müssen, was zunächst einen zusätzlichen Arbeitsschritt
30 darstellt.

35

Die Abtrennung der Emulsion erfolgt entweder durch die
natürliche Trennung zweier Phasen, durch Zentrifugieren
oder durch Zusatz eines emulsionbrechenden Mittels.
Während im ersten Fall nicht sichergestellt ist, daß
Restbestände der Emulsion in dem zu reinigenden System
zurückbleiben, wird im zweiten Fall das gesamte System
hohen Zentrifugalkräften unterzogen, die insbesondere

Correspondence
GPO Box 1074
Brisbane QLD 4001
Australia

Offices
Brisbane
Gold Coast

ABN 16 251 059 175

Level 26, MLC Building
239 George Street, Brisbane
QLD 4000 Australia
Telephone +61 7 3011 5555
+61 7 3221 8761
Facsimile +61 7 3229 3384
+61 7 3229 6598
Email mail@cullens.com.au
Web Site www.cullens.com.au

CLAUDE ANNE
RONALD A. HALLIDAY
JAN & JONGE
KENNETH G. HINNEY

ALISON M. McHILLAN
ELINA MCCUTCHEON
BARRY JAMES
WENDY DEAR

GINT SHINS
DAVID MORGAN
BRENT ELLIS

Patents and Trade Mark Attorneys: Brisbane, Sydney, Melbourne, Perth, Adelaide, Hobart, Auckland, Wellington, Christchurch, Dunedin, Invercargill, Auckland, New Zealand.



CULLEN & CO.

— Patent & Trade Mark Attorneys —

3310263

1 bei biologischen Flüssigkeiten, wie Blut, zur Zerstörung
der Blutkörperchen führen. Auch der Einsatz von emul-
sionsbrechenden Mitteln ist bei biologischen Flüssigkei-
ten nicht angebracht, da diese selbst im wesentlichen
5 toxisch sind und somit für diese Zwecke nicht eingesetzt
werden können.

Auch die natürliche Trennung der Emulsion von einem wäss-
rigen System hat sich gerade bei biologischen Flüssigkei-
ten als nicht durchführbar erwiesen, da die Folgeerschei-
nungen nicht zu übersehen sind, wenn derartige Flüssig-
keitsmembran-Emulsionen direkt mit Blut in Berührung ge-
bracht werden und evtl. Restbestände der die Flüssigmem-
bran bildenden Flüssigkeit im Blut zurückbleiben.

15 Demzufolge liegt der Erfindung die Aufgabe zugrunde, ein
Verfahren der eingangs erwähnten Art zu schaffen, mit
dem kontinuierlich lipophile Stoffe aus einem wässrigen
System entfernt werden können, ohne daß eine Vermischung
20 des wässrigen Systems mit der zu extrahierenden Flüssig-
keit stattfindet.

Weiterhin liegt der Erfindung die Aufgabe zugrunde, eine
Vorrichtung zur Verfügung zu stellen, mit der das vor-
stehende Verfahren durchführbar ist.

Diese Aufgaben werden durch die Erfindung gelöst.

30 Gegenstand der Erfindung ist ein Verfahren zur Entfernung
von lipophilen Stoffen aus wässrigen Lösungen, insbeson-
dere aus biologischen Flüssigkeiten, bei dem die zu rei-
nigende Lösung und die Reinigungsflüssigkeit durch eine
Membran getrennt sind und an dieser vorbeigeführt werden
und die dadurch gekennzeichnet ist, daß man als Reini-
35 gungsflüssigkeit ein lipophiles Lösungsmittel einsetzt.

Das erfindungsgemäße Verfahren weist zunächst im wesent-
lichen das gleiche Trennverhalten wie die bekannte

Correspondence
GPO Box 1074
Brisbane QLD 4001
Australia

Offices
Brisbane
Gold Coast

ABN 16 251 059 175

Brisbane Office
Level 20, MLC Building
239 George Street, Brisbane
QLD 4000 Australia

Telephone +61 7 3011 5555
+61 7 3221 8761
Facsimile +61 7 3229 3384
+61 7 3229 6598

Email
mail@cullens.com.au
Web Site
www.cullens.com.au

HELMUT A. EICHERBERGER *
CLAUDIO ANESE *
RONALD A. HALIDAY *
IAN de JONGE *
KENNETH G. FINNEY *

ALISON M. MILLAN *
MIRACI EPTA
ELISA McCUTCHEON *
HLE BN EPTA
BARRY JAMES
WENDY DEAR
MILLIE Hous PULL

GINT SHINS
DAVID MORGAN
IRENE ELIUI
RUE Practice Manager

Patent and Trade Mark Attorneys - Australia and New Zealand - Legal Practitioners - Foreign & Australian

11
6

1 Flüssigmembrantechnik auf, ohne jedoch dessen Nachteile
zu besitzen. Es werden also hochselektiv lipophile Stoffe
aus wässrigen Lösungen abgetrennt und aus dem gesamten
System entfernt.

5

Es weist gegenüber der Flüssigmembrantechnik den Vorteil
auf, daß keine Emulsionen hergestellt werden müssen, daß
also die Einverleibung der Reinigungsflüssigkeit in eine
Flüssigmembranphase entfällt und auch keine Emulsionen
10 mit der zu reinigenden Lösung vermischt werden müssen.
Damit entfällt auch eine Abtrennung der Emulsion von dem
zu reinigenden System, so daß keine schädlichen Wirkungen
auftreten können.

15

Das erfindungsgemäße Verfahren wird folgendermaßen durch-
geführt:

Die zu reinigende wässrige Lösung, beispielsweise Körper-
flüssigkeiten, wie Blut, wird an einer polymeren Membran
20 entlanggeführt, wobei es möglich ist, eine Membran mit
polaren oder unpolaren Eigenschaften einzusetzen. Dieser
Verfahrensschritt unterscheidet sich im wesentlichen
nicht von der Flüssigkeitsführung auf der Blutseite bei
der Hämodialyse oder Hämofiltration.

25

Auf der anderen Seite der Membran wird jedoch im Gegen-
satz zur Hämodialyse, bei der ein wässriges System ein-
gesetzt wird, als Reinigungsflüssigkeit ein im wesentli-
chen lipophiles Lösungsmittel eingesetzt, dessen Lösungs-
30 vermögen für lipophile Stoffe erheblich über dem von Was-
ser liegt.

35

An der hydrophoben Membran entsteht durch das Vorbeilei-
ten unterschiedlicher Flüssigkeiten eine Phasengrenz-
schicht, da die Membran eine Barriere darstellt und in
einer bevorzugten Ausführungsform die beiderseitig vor-
liegenden Flüssigkeiten ineinander im wesentlichen nicht
lösbar sind. Aufgrund des vorliegenden Konzentrations-

- 1 gefälles permeieren die im wässrigen System, beispiels-
weise Blut, vorliegenden lipophilen Substanzen, beispiels-
weise die vorstehend genannten Lebertoxine, durch die
hydrophobe Membran und durch die Phasengrenzschicht und
5 werden von der Reinigungsflüssigkeit aufgenommen, die die-
se Stoffe erheblich besser solvatisiert als die wässrige
Lösung.

- 10 Anschließend wird die Reinigungsflüssigkeit entweder so-
lange im Kreis geführt, bis ihre Aufnahmefähigkeit für
die lipophilen Substanzen erschöpft ist, also das Konzen-
trationsgefälle zwischen den beiden Flüssigkeiten ausge-
glichen ist, und anschließend ausgetauscht oder aber wäh-
rend der Extraktion der lipophilen Substanzen stetig von
15 diesen befreit, beispielsweise durch Adsorption dieser
Substanzen an entsprechenden Adsorbenzien, elektrochemi-
sche Abtrennung, chemische Umsetzung oder Ausfällung die-
ser Substanzen u.dgl.

- 20 Nach der Behandlung mit dem erfindungsgemäßen Verfahren
ist die zu reinigende Flüssigkeit im wesentlichen von den
abzutrennenden lipophilen Stoffen befreit und kann
wunschgemäß wieder eingesetzt werden.

- 25 Es spielt dabei, wie vorstehend erläutert, keine nennens-
werte Rolle, welche Polaritätseigenschaften eine Membran
besitzt, sofern sichergestellt ist, daß wenigstens eine
der beiden Flüssigkeiten die Membran benetzt. Da im Re-
gelfall Wasser als polares Lösungsmittel auf der Seite
30 der zu reinigenden Lösung und ein unpolares Lösungsmit-
tel, das in Wasser im wesentlichen nicht lösbar ist, vor-
liegen, wird eine dieser Flüssigkeiten die Membran be-
netzen, so daß die Membranöffnungen durch eines der bei-
den Lösungsmittel gefüllt ist. Da die benetzende Flüssig-
35 keit zugleich in aller Regel in einem dünnen Film auf
die unmittelbar der anderen Flüssigkeit zugewandten Ober-
fläche der polymeren Membran aufziehen wird, stehen die
beiden Flüssigkeiten in Form einer im wesentlichen zwei-

1 dimensionalen Grenzschicht unmittelbar in Berührung, so
daß die zu extrahierenden lipophilen Stoffe aus der wass-
rigen Lösung in die Reinigungsflüssigkeit diffundieren
und somit entfernt werden können.

5 Nach der Reinigung kann die Membran bzw. ein aus einer
Vielzahl von Membranen hergestelltes Filter wie die Rei-
nigungsflüssigkeit weggeworfen werden, ohne daß es einer
speziellen Aufbereitung bedürfte.

10 Weitere Einzelheiten, Merkmale und Ausführungsformen sind
in der Zeichnung unter Bezugnahme auf die Beschreibung
erläutert.

15 Es zeigen

Fig. 1 eine schematische Darstellung der Reinigungseinheit
der Erfindung

20 Fig. 2 einen vergrößerten Ausschnitt aus der Reinigungs-
einheit unter Darstellung der benetzten Membran

Fig. 3 einen weiteren vergrößerten Ausschnitt aus der
Reinigungseinheit gemäß der Erfindung unter
Herausstellung der benetzten Membran
und

25 Fig. 4 eine schematische Ansicht der erfindungsgemäßen
Vorrichtung zur Reinigung von wässrigen Lösungen.

30 Zu den in wässrigen Lösungen gelösten Stoffen, die nach
dem Verfahren der Erfindung abgetrennt werden können,
gehören im wesentlichen lipophile Stoffe, die anorgani-
scher oder organischer Art sein können. Unter lipophilen
Stoffen werden auch solche Stoffe verstanden, die glei-
chermaßen in polaren und unpolaren Flüssigkeiten löslich
35 sind. Es sind sogar solche Stoffe darunter zu verstehen,
die erheblich besser in Wasser löslich sind als in un-
polaren Lösungsmitteln, jedoch noch in den letzteren
eine begrenzte Löslichkeit besitzen. Die Grenze ist je-

1 doch dann erreicht, wenn bei der Durchführung des erfindungsgemäßen Verfahrens praktisch keine nennenswerte Extraktion der zu extrahierenden Stoffe mehr stattfindet. Dabei spielt es erfindungsgemäß keine wesentliche Rolle, 5 ob diese Stoffe neutral, sauer oder basisch sind, sofern sie in der Reinigungsflüssigkeit zumindest im geringen Umfang löslich sind.

Bei Verwendung von Blut als zu reinigender Phase, beispielsweise zur Abtrennung der beim Lebersversagen auftretenden Toxine oder von dem Blut gelösten Arzneimitteln, wird man als Reinigungsflüssigkeit eine solche Flüssigkeit wählen, die einerseits die Toxine wenigstens etwas zu solvatisieren vermag, andererseits jedoch für den Patienten unschädlich ist und das Blut nicht angreift. Insbesondere werden solche Flüssigkeiten eingesetzt, die ein erheblich besseres Lösungsvermögen gegenüber den zu extrahierenden Stoffen aufweisen als das Blut selbst und überdies aus pharmakologischen Gesichtspunkten unbedenklich sind. Besonders bevorzugt sind als Reinigungsmittel der eben erwähnten Art solche Lösungsmittel, die in Wasser nicht löslich sind. Unter in Wasser nicht löslichen Lösungsmitteln werden solche Lösungsmittel verstanden, die in Wasser höchstens zu 1 - 2 Vol.-% löslich sind. Hierzu gehören höherkettige Kohlenwasserstoffe, beispielsweise Paraffine oder Isoparaffine, halogenierte Kohlenwasserstoffe, Ether, höhere oxigenierte Verbindungen, wie Alkohole, Ketone, Säuren und Ester. Weiterhin können hierfür Siliconöle, Öle pflanzlichen und tierischen Ursprungs, Naphtene und Aromaten mit einem Molekulargewicht bis 1000 verwendet werden. 10 15 20 25 30

Bevorzugt sind für die Anwendung beim Menschen stark raffinierte Mineralöle, zu denen auch die Paraffinkohlenwasserstoffe gehören. Weiterhin können Öle pflanzlichen und tierischen Ursprungs, wie Sojabohnenöl, Baumwollsaatöl u.dgl. eingesetzt werden. Diese Öle können auch im stark hydrierten Zustand in vorteilhafter Weise 35

Clin Biochem 1981 Jun;14(3):119-25

Quantitation of lipid profiles from isolated serum lipoproteins using small volumes of human serum.

Bloom RJ, Elwood JC

Methodology is described that isolates the individual serum lipoproteins, VLDL, LDL and HDL and quantitates the free cholesterol, esterified cholesterol, triglycerides and phospholipid classes in each fraction using 2-3 mL of serum. The determination of the methyl esters of fatty acids from the various lipid classes is described. The lipoproteins are isolated by non-linear density ultracentrifugation using 1 mL of serum per swinging bucket. The lipids are obtained by solvent extraction. The cholesterol, cholesterol esters and triglycerides are separated by TLC using a petroleum ether:diethyl ether system and the phospholipids are separated using a chloroform:methanol system. All lipid classes are quantitatively determined and recovery data are presented. Analysis of the fatty acid profiles of the lipid classes using GLC is described. The methodology can be adapted to partial determination if in-depth studies are not required.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.